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Endothelial Function Progressively Deteriorates During Normal Pregnancy

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ABSTRACT

Objective. To elucidate changes in endothelial function throughout the gestational period in normal pregnancy and its relationship with plasma sFlt-1 levels. *Methods.* Endothelial function was evaluated by reactive hyperemia index (RHI) using Endo-PAT2000 and plasma sFlt-1 levels were measured simultaneously by ELISA. *Results.* RHI gradually deteriorated with increasing the gestational age. Plasma sFlt-1 levels exhibited a gradual increase at late pregnancy and were inversely correlated with RHI. *Conclusion.* Maternal endothelial function gradually deteriorates with increasing gestational age and there is an inverse correlation between endothelial function and plasma sFlt-1 levels in normal pregnancy.

Keywords Peripheral arterial tonometry, Pregnancy, Endothelial dysfunction, sFlt-1, Preeclampsia.

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INTRODUCTION

In normal pregnancy, blood volume and cardiac output increase as pregnancy proceeds. By contrast, blood pressure gradually decreases slightly toward mid-pregnancy and then slowly increases toward the term (1). It is assumed that this discrepancy is due at least in part to alterations in endothelial function based on reductions in systemic vascular resistance by nitric oxide (NO) (2). In preeclampsia (PE), it is acknowledged that poor placentation in early pregnancy causes placental hypoperfusion with subsequent maternal endothelial dysfunction. The endothelial dysfunction in various tissues is implicated in symptoms such as hypertension, proteinuria, tissue oedema, cerebral haemorrhage, and so on (3). Therefore an assessment of endothelial function might be useful for prediction, prevention, and curative strategies for PE patients. However, it is still controversial whether endothelial function changes throughout normal pregnancy (4-8).

Endothelial function includes vasodilatory, anti-coagulant, anti-inflammatory and vascular permeability activities. It is thought that vascular homeostasis is well maintained (9) and NO and vascular endothelial growth factor (VEGF) play central roles in these mechanisms. Soluble fms-like tyrosine kinase-1 (sFlt-1) has been implicated in the symptoms of PE. It is a soluble type of VEGFR-1 and competes with VEGF for binding to VEGF receptors. Rats over-expressing sFlt-1 show similar clinical features of PE (10). In PE patients, increased circulating sFlt-1 levels have been reported compared with normal pregnant women

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(10, 11) and the importance of elevated sFlt-1 levels has been discussed in relation to endothelial dysfunction (12). However, the relationship between circulating sFlt-1 levels and endothelial function in normal pregnancy remains unclear.

To assess endothelial function, flow-mediated dilation (FMD) of the brachial artery during reactive hyperemia (RH) has been introduced. Though FMD is a gold standard for non-invasive assessment of endothelial function, the measurement of FMD has a potential limitation regarding its intra-and inter-operator reproducibility (13). To overcome this problem, peripheral arterial tonometry (PAT) has recently been developed. The Endo-PAT2000 system (Itamar Medical, Caesarea, Israel) does not require the operator to have the training and specialized skill needed when using ultrasonography. Corretti reported that PAT method is significantly correlated with FMD ($p < 0.0001$) (13). In comparing PAT with FMD, Carty measured PAT and FMD in the same pregnant women. In the result, it was showed that both parameters compared favorably (14). The data reflect arterial tone alterations in the peripheral microvascular bed and can be digitalized and read as reactive hyperemia index (RHI). The influence of the autonomic nervous system can be eliminated by measuring the contralateral arm as a control. Thus, the PAT method might be superior to FMD in terms of convenience, quantitation, and reproducibility (15). RHI in patients with coronary endothelial dysfunction was reported to be relatively lower (16). Another study demonstrated that administration of L-NAME, known as an inhibitor of eNOS, reduced RHI, implicating NO-

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dependent endothelial function (17). In addition, Aragones reported that elevated levels of sE-selectin, a biomarker for endothelial dysfunction, were associated with lower RHI (18). However there have been few articles so far investigating the role of endothelial function using RHI in PE patients. One report presented reduced RHI in patients with PE compared with normal pregnant women around 32 weeks of gestation (19). Recently, Carty et al. demonstrated that RHI in pregnant women at 28 weeks was significantly lower than that at 16 weeks (14), but they just focused on the predictive value of the onset of PE and concluded that it failed for that purpose.

Here we report a prospective study of monitoring maternal RHI to evaluate endothelial function throughout the normal gestational period using the Endo-PAT system. We also measured endothelial function and circulating sFlt-1 levels in non-pregnant women, preeclamptic patients and pregnant women who develop PE later. Because the significance of increased sFlt-1 levels in normal late gestation has not been clarified, we measured plasma sFlt-1 levels in all subjects and evaluated the relationship between RHI and plasma sFlt-1 levels. The aim of this study was to elucidate alterations in endothelial function using the PAT system and its correlation to plasma sFlt-1 levels throughout the normal gestational period as well as the process of PE.

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MATERIALS AND METHODS

Study Population

All pregnant women consulting our department at Kyoto University Hospital between March 2011 and March 2012 were considered for enrolment. Complicated cases with chronic hypertension, diabetes mellitus, chronic kidney disease, autoimmune diseases like systemic lupus erythematosus and respiratory diseases were excluded. Over eighty percent of eligible pregnant women were enrolled in the present study. Age-matched, apparently healthy non-pregnant women were also enrolled. In addition, we enrolled 7 patients who had already developed symptoms of PE and had been treated with antihypertensive drugs. The protocol was approved by the institutional ethics committee of Kyoto University Graduate School of Medicine. Written informed consent was obtained from all subjects.

Measurement of Endothelial Function

PAT is a new technique to non-invasively assess endothelial function. It comprises finger probes to evaluate digital volume changes accompanied by pulse waves (20). We used the Endo-PAT2000 system to evaluate endothelial function. All participants were asked to abstain from caffeine, alcohol and cigarette smoking for at least 24 hours before the examination. The PAT study was conducted at least 3 hours after a light meal. A temperature

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controlled (23 to 26 ℃), quiet and dark room was reserved for the examination. A blood pressure cuff was placed on one upper arm and a probe was placed on the tip of the index finger; the probe sensor on the contralateral arm served as a control. Following approximately 15 min of resting in a supine position on the bed, the baseline digital volume signals were recorded for 5 min, then the blood pressure cuff was inflated to 200 mmHg to occlude the brachial artery and the pressure was maintained for 5 min. The cuff was then released to induce RH and the reaction was recorded for 5 min. The analytical method of calculating RHI was as follows. The RHI reflects the ratio of the alteration in the amplitude from 90 to 150 seconds after the cuff is released (occluded arm –A; non-occluded arm - C) divided by the average amplitude of the signal from 170 to 20 seconds before the cuff is inflated (occluded arm - B; non-occluded arm - D). This value was calculated using the formula: RHI = (A/B) / (C/D). The RHI data were digitally analysed using the Endo-PAT2000 software version 3.3.2 (Itamar Medical). Each pregnant woman underwent this test 1 to 3 times at intervals of longer than one month apart.

Plasma Sampling and Measurement of sFlt-1 Levels

Plasma samples were collected from all pregnant women who participated in this study and age-matched non-pregnant women at the same day as the PAT examination. The blood was collected into tubes containing EDTA and the plasma was then separated by

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centrifugation at 3000 rpm for 30 min. The supernatants were stored at -20 ℃ until analysis. Plasma sFlt-1 levels were measured using a human sVEGFR1/Flt-1 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) in duplicate according to the manufacturer's protocol. Inter-assay and intra-assay coefficients of variation were 5.5–9.8 % and 2.6–3.8 %, respectively.

Diagnosis of PE

PE was defined as the new onset of hypertension and proteinuria after 20 weeks of gestation. Hypertension was defined as a systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg on two measurements at least 4 hours apart, and proteinuria as ≥ 300 mg/day (21). In Japan, it is defined that late-onset preeclampsia develops at or after 32 weeks of gestation.

Statistical Analysis

The results of normally distributed continuous variables are expressed as the mean ± SEM (range), while those with skewed distribution are expressed as the median value with [interquartile range]. Continuous variables were analysed by the Wilcoxon *t* test, Mann-Whitney *U* test and Kruskal-Wallis *H* test, as appropriate. Pearson's correlation coefficient was used to evaluate possible associations. A p value of < 0.05 denoted statistical significance.

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Statistical analyses were performed using Prism 3.0 (GraphPad Software, La Jolla, CA).

RESULTS

Patient Characteristics

The information collected on the study population is shown in Table 1. Three of 29 pregnant women developed PE. Body mass indexes were significantly higher in the normal control pregnant women than in the non-pregnant women. In 7 PE patients, systolic and diastolic blood pressures were not high due to antihypertensive treatment and the median time of measuring RHI was 32 [31.5–34] weeks of gestation. The average of systolic blood pressure was 161 ± 5 mmHg, and the diastolic blood pressure was 95 ± 4 mmHg, when PE patients were diagnosed at first time. RHI measurements in PE patients were performed 4 [2.5-5.5] days after start of the treatment. Gestational age at delivery was earlier in the PE group than in normal pregnant women. Neonatal weight was also lighter in the PE patients than in normal pregnant women and patients with late PE development.

Alterations of RHI in Normal Pregnancy

The RHI gradually decreased throughout gestation in normal pregnant women (Figure 1A). The RHI in weeks 20–31 and over 32 weeks of gestation was significantly lower than that prior to 20 weeks of gestation. However, there was no significant difference in RHI

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between non-pregnant women and pregnant women under 19 weeks of gestation (Figure 1B).

Relation of RHI to Plasma sFlt-1 Levels

Plasma sFlt-1 levels gradually increased throughout gestation in normal pregnant women (Figure 2A). They were higher in pregnant women over 32 weeks of gestation than those at earlier periods of gestation (Figure 2B). The RHI was inversely correlated with plasma sFlt-1 levels ($p = 0.0074$) for all 56 measurements in normal pregnant women (Figure 3).

Figure 4 shows comparisons of RHI (A) and plasma sFlt-1 levels (B) during 3 gestational periods (≤ 19 weeks, 20–31 weeks, ≥ 32 weeks). Nine normal pregnant women were given repeat measurements in each gestational period. The RHI in pregnant women over 32 weeks of gestation was significantly lower than in those under 19 weeks of gestation. In contrast, plasma sFlt-1 levels in pregnant women over 32 weeks of gestation were significantly higher than those less than 20 weeks or between 20 and 31 weeks of gestation.

RHI and Plasma sFlt-1 Levels in PE and Late PE Development Patients

The RHI in PE patients was significantly higher than in normal pregnant women at the same gestational stage (31–36 weeks) (Figure 5A). On the other hand, plasma sFlt-1 levels showed over a 3-fold increase in PE patients ($p = 0.004$) (Figure 5B). RHI was measured in two PE patients after vaginal delivery. The RHI in one case was increased (1.58 to 1.86) nine

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days after the delivery. In another case, it was similarly increased (1.65 to 1.84) twelve days after the delivery.

Three of 29 pregnant women developed PE in this study. The RHI and plasma sFlt-1 levels were measured at 10–15 weeks and 29–32 weeks of gestation, before the onset of PE in these 3 patients. Both RHI and plasma sFlt-1 levels were no different at 10–15 weeks of gestation. While RHI was still comparable at 29–32 weeks of gestation, plasma sFlt-1 levels were slightly higher in patients with late PE development.

COMMENT

Salient findings of the present study were as follows: (1) Endothelial function evaluated by PAT gradually deteriorated with the progression of gestational age in normal pregnant women. (2) Plasma sFlt-1 levels exhibited a gradual increase late in pregnancy and were inversely correlated with the RHI. (3) In the PE patients, RHI, an index of endothelial function, showed a higher value in the presence of higher plasma sFlt-1 levels compared with normal pregnant women at similar gestational ages.

In the present study, we examined endothelial function by PAT in more detail and demonstrated for the first time that RHI gradually decreases with increasing gestational age. While some reports have claimed endothelial function ameliorates, others reported a decline throughout normal pregnancy (4-8). These earlier studies utilized FMD of the brachial artery

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as a method of evaluating endothelial function. Lack of good reproducibility in the FMD technique may explain, at least in part, inconsistent results in earlier studies. On the other hand, the PAT technique provides more accurate information on endothelial function regardless of the operator's skill and the autonomic nervous system activity of the examinee. (15). Carty et al. has used PAT and reported that RHI at 28 weeks of normal pregnancy was significantly lower than at 16 weeks (14). In the present study, we examined endothelial function by PAT in more detail and demonstrated that RHI gradually decreases with increasing gestational age, consistent with recent reports (14). Therefore we conclude that endothelial function was deteriorating even in normal pregnancy.

Although it has been already shown that sFlt-1 levels are increasing during gestation, we first that demonstrated that plasma sFlt-1 levels and RHI were inversely correlated. Until now, there is no study showing a correlation between endothelial function and plasma sFlt-1levels in normal pregnant women. As one of the reasons, we think that data analysis by FMD method may have some difficulty, in terms of convenience, quantitation, and reproducibility. Increased plasma sFlt-1 are suggested to neutralize VEGF and attenuate the beneficial effect of VEGF on endothelial function in PE patients, though the relation between plasma sFlt-1 levels and endothelial function remains unclear in normal pregnancy. From the current results, it can be speculated that the elevation of plasma sFlt-1 levels in the late pregnancy may cause endothelial dysfunction. However, it is unknown whether elevated sFlt-1 influences

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endothelial function directly and further studies will be needed to elucidate this relationship.

In our findings, the RHI in PE patients was relatively high. Markedly increased plasma sFlt-1 levels in PE patients strongly suggest poorer endothelial function (22). It has already been demonstrated that FMD is reduced in PE patients (23-25). Therefore our results appear curious and must be interpreted with caution. The following two reasons may account for about the cause of dissociation of this result. First, all PE patients in our study were medicated with calcium blocker and/or magnesium sulfate, which potentially improved their endothelial function. Second, there is a report that RH itself is slightly pressure-dependent ($\beta = 0.007$, $p < 0.001$) (26). Generally, it is thought that endothelial dysfunction leads to hypertension and arteriosclerosis. However, in the presence of high blood pressure, an evaluation of endothelial function by PAT may be influenced by blood pressure. In PAT analysis, in contrast to our result showing increased RHI, Yinon et al. (19) have reported decreased RHI in a study in PE patients in which cases of relatively early onset were enrolled more frequently than in ours. It is probable that markedly depressed endothelial function leads to a lower RHI despite the presence of high blood pressure. Thus the relatively high RHI values in PE patients might be due to their higher blood pressure level or to drugs, though this needs to be further elucidated.

The limitation to the present study was that the number of patients who developed PE at the later gestational stage was too small for us to be able to assess whether endothelial function in the early stages of gestation is able to predict the onset of PE.

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Angiogenic factors, including VEGF, in maternal vessels are important for maintaining normal endothelial function. In PE patients, the normal balance between angiogenesis and anti-angiogenesis has been compromised by circulating factors, including sFlt-1 and others, from the placenta. Up to the present, there is no method to evaluate endothelial function that combines non-invasiveness with convenience, quantitation and reproducibility. Although further study must be done regarding the influence of blood pressure levels, it appears that PAT will become a useful tool to assess maternal endothelial function hereafter. We believe research into predicting or understanding the pathogenesis of PE might progress if more measurements of anti-angiogenic or angiogenic factors in maternal blood can be added to the accumulating data on this topic.

In conclusion, we here evaluated maternal endothelial function using the novel methodology of the Endo-PAT2000 system and simultaneously measured plasma sFlt-1 levels in pregnant women. Our investigation revealed that maternal endothelial function gradually deteriorated while plasma sFlt-1 levels increased in inverse correlation with gestational age in normal pregnancy. Anti-angiogenic or angiogenic factor profiling combining with RHI will be valuable for predicting and understanding the pathogenesis of PE.

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Declaration of Interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Figure Legends

Figure 1: Reactive hyperemia index (RHI) of the patients. A: RHI throughout the normal gestation (n = 56). B: Comparison of RHI among 3 gestational periods (≤ 19 weeks, 20-31 weeks, ≥ 32 weeks) of normal pregnant women and non-pregnant women. **: $p < 0.001$

Figure 2: Plasma sFlt-1 levels of the patients. A: Plasma sFlt-1 levels throughout the normal gestation (n = 56). B: Comparison of plasma sFlt-1 levels among normal pregnant women and non-pregnant women. a: $p < 0.05$ vs. non-pregnant women, b: $p < 0.05$ vs. ≤ 19 weeks of gestation, c: $p < 0.05$ vs. 20-31 weeks of gestation.

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Figure 3: Correlation of reactive hyperemia index to plasma sFlt-1 levels (n = 56).

Figure 4: The change of reactive hyperemia index (A) and plasma sFlt-1 levels (B) in serial 3 gestational periods in the same patients (n = 7).

Figure 5: Comparisons of reactive hyperemia index (A) and plasma sFlt-1 levels (B) between PE (n = 7) and normal pregnant women (n = 17) at the same gestational age (31-36 weeks of gestation).

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Table1: Clinical characteristics and pregnancy outcomes in study subjects.

	Non-pregnant women	Normal pregnant women	Late PE development	PE
Subjects (n)	9	26	3	7
Total number of measurement (n)	9	56	6	7
Age (years)	32.0 ± 2.4 (21-45)	33.3 ± 1.0 (25-41)	36.0 ± 2.0 (34-40)	34.6 ± 0.6 (24-36)
Primipara (n)	(-)	23/26	1/3	5/7
Physical findings at the first time of measurement:				
Body mass index (kg/m^2)	19.3 ± 0.5 (16.6-21.4)	24.1 ± 0.9 [a] (18.4-37.9)	21.7± 1.4 (18.8-23.1)	26.4 ± 1.4 [a] (23.1-32.0)
Systolic blood pressure (mmHg)	110 ± 2 (98-120)	108 ± 2 (90-133)	108 ± 6 (100-119)	136 ± 5 [a, b] (120-150)
Diastolic blood pressure (mmHg)	66 ± 3 (52-75)	63 ± 2 (46-74)	65 ± 4 (60-72)	87 ± 3 [a, b, c] (76-96)
Outcomes:				
Gestational age at delivery (weeks)	(-)	39 [38-40]	37 [37-37.5]	35 [b] [34-37]
Neoatal weight (g)	(-)	2986 [2852-3078]	2866 [2643-2998]	1986 [b,c] [1805-2437]
SGA (n)	(-)	0/26	0/3	2/7

PE: preeclampsia, SGA: small for gestational age. Values are the mean ± SEM and (range) or median value with [interquartile range]. a: $p < 0.05$ vs. non-pregnant women, b: $p < 0.05$ vs. normal pregnant women, c: $p < 0.05$ vs. late PE development

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Figure 1 (K. Fujita)

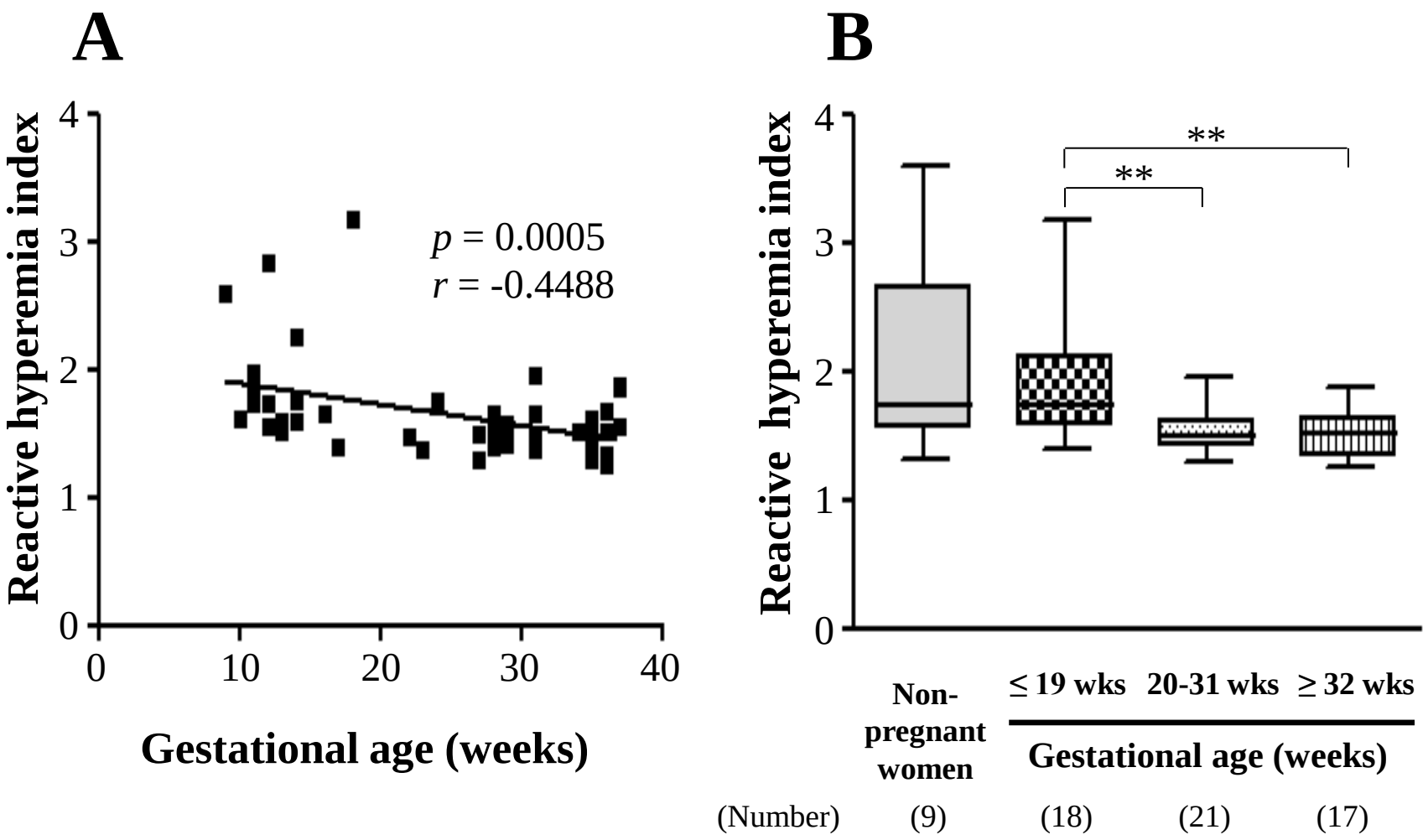

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Figure 2 (K. Fujita)

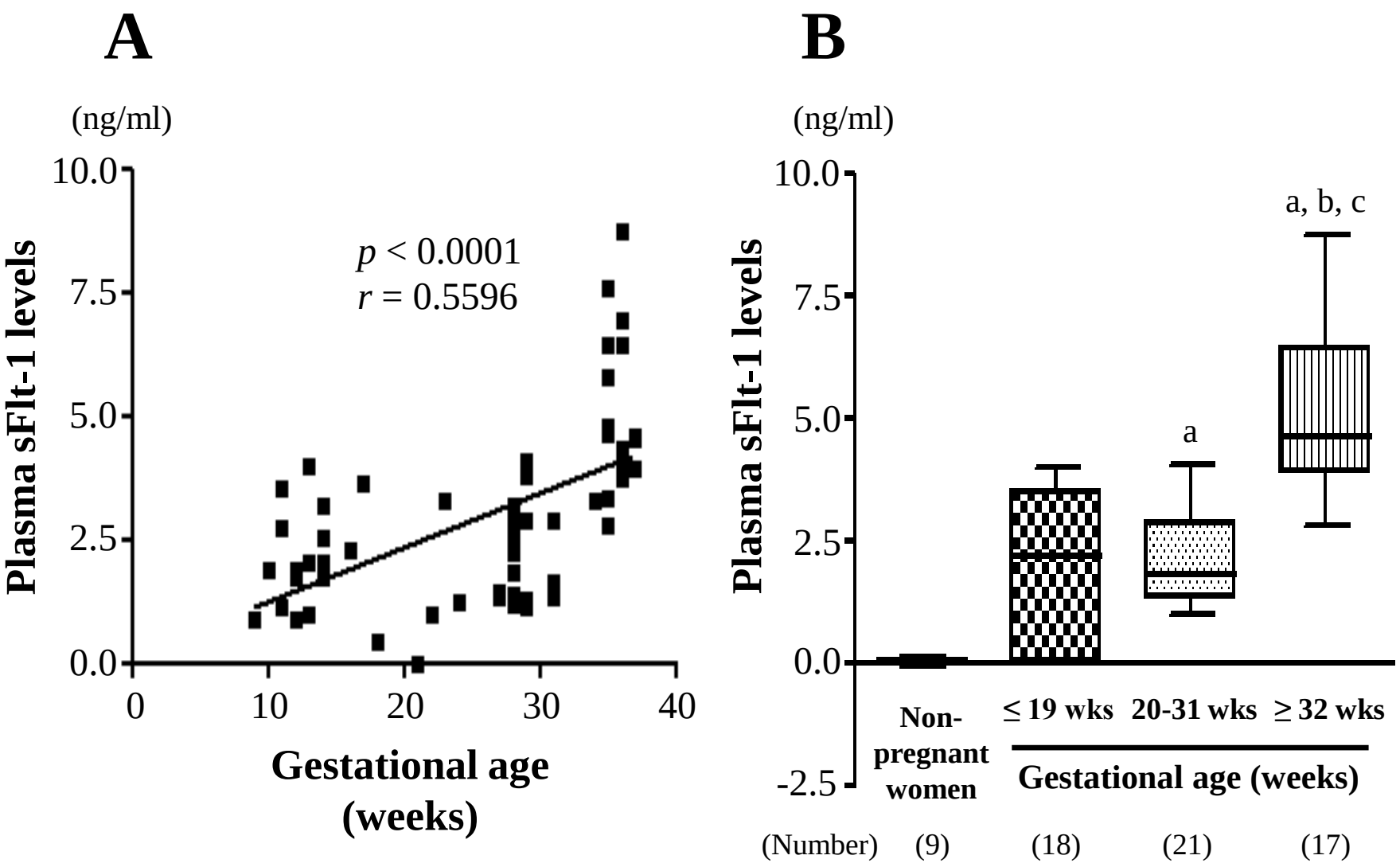

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Figure 3 (K. Fujita)

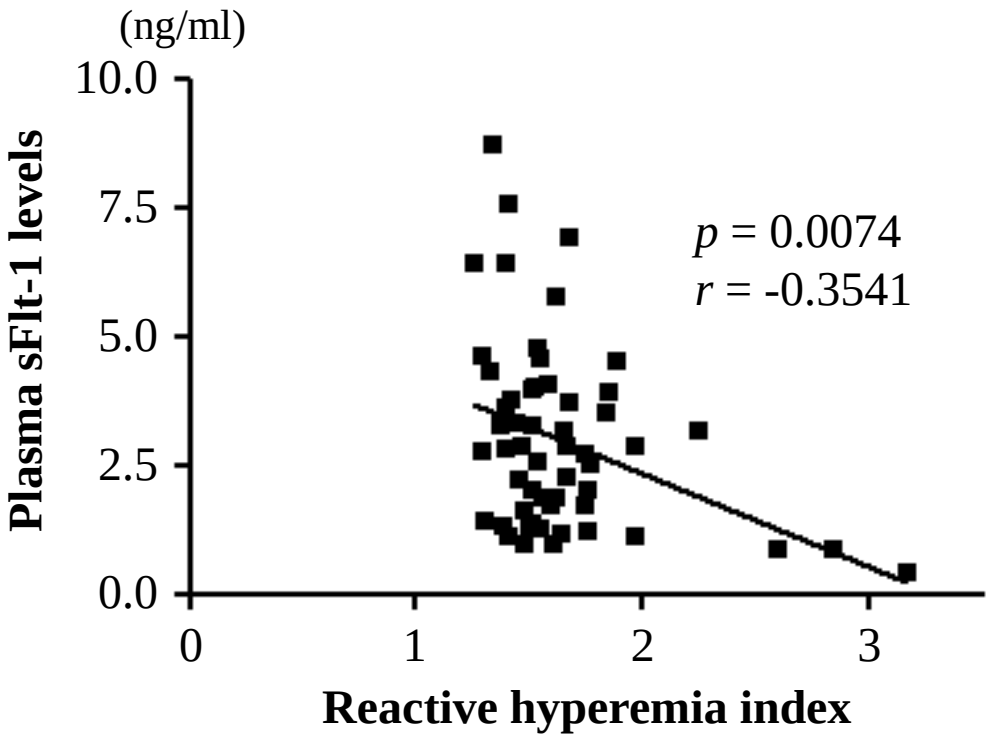

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Figure 4 (K. Fujita)

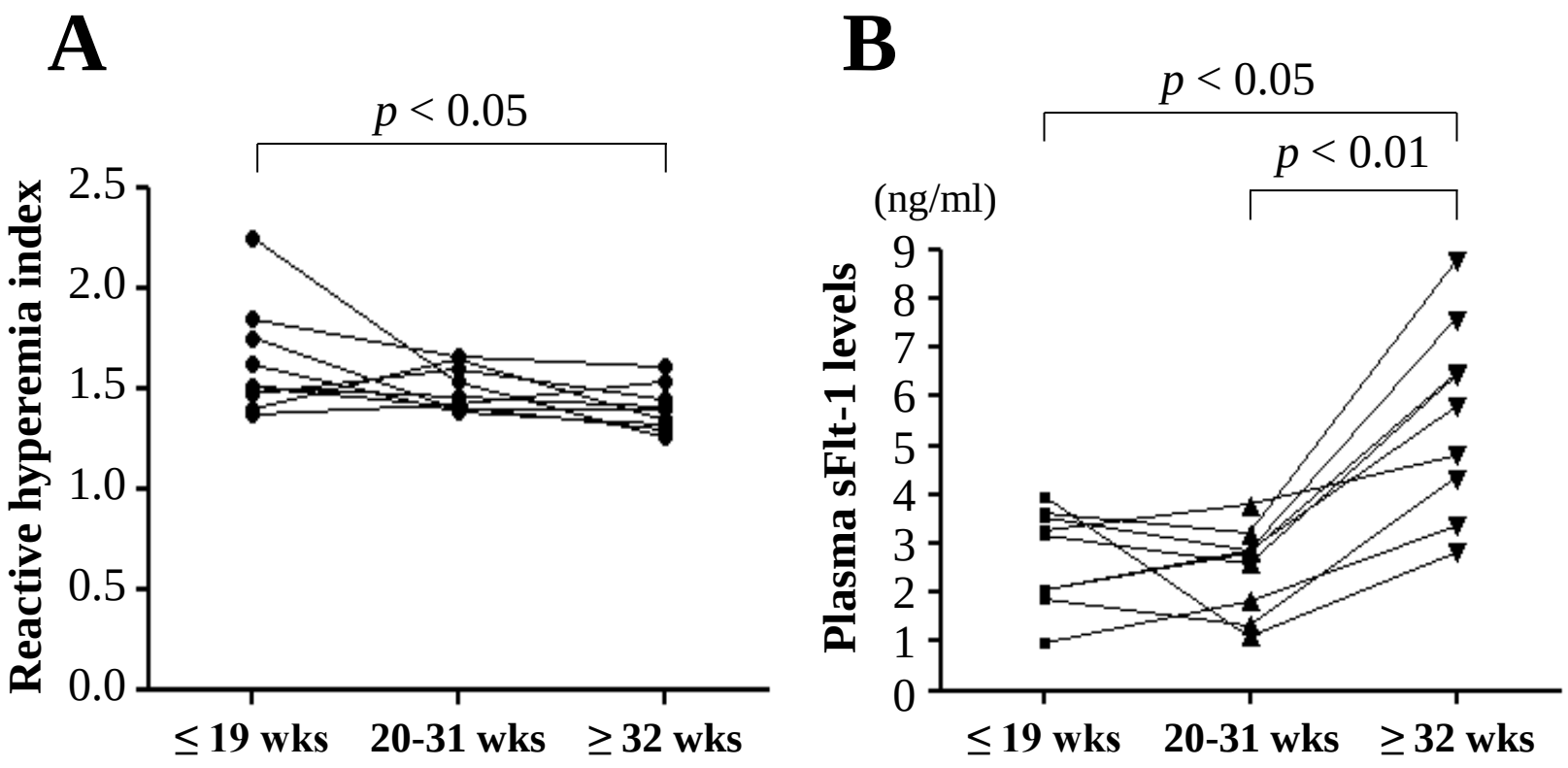

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Figure 5 (K. Fujita)